



EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions

UPDATE OF THE OPINION ON
TSE INFECTIVITY DISTRIBUTION IN RUMINANT TISSUES

INITIALLY ADOPTED BY
THE SCIENTIFIC STEERING COMMITTEE
AT ITS MEETING OF 10-11 JANUARY 2002
AND AMENDED AT ITS MEETING OF 7-8 NOVEMBER 2002

**following the submission of (1) a risk assessment by the German Federal Ministry of
Consumer Protection, food and Agriculture and (2) new scientific evidence
regarding BSE infectivity distribution in tonsils**

OPINION

At the end of 2001, the Scientific Steering Committee (SSC) was invited:

- (1) To update, on the basis of the most recent scientific data, the sheep tissue infectivity titre table presented in the SSC opinion of 22-23 July 1999 on The Policy of Breeding and Genotyping of Sheep;
- (2) To create a similar table for cattle on the basis of all available scientific evidence;
- (3) To consider whether any new evidence exists since the adoption of its opinion of 9 December 1997 on the listing of Specified Risk Materials which would indicate that the entire head of cattle, sheep and goats, including skeletal muscle, tongue and associated innervation should be considered as specified risk material.

The SSC invited the TSE/BSE *ad hoc* Group to prepare a scientific report that could serve as the basis for preparing an answer to the above question. A first report was finalised by the TSE/BSE *ad hoc* Group at its meeting of 13 December 2001 it served as the basis for an opinion adopted by the SSC at its meeting of 10-11 January 2002.

On 10 May 2002, the Government of the Federal Republic of Germany requested the European Commission to elicit the opinion of the Scientific Steering Committee on a report of 25 April 2002 prepared by the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV), which evaluates the possible risks related to harvesting cheek meat and more specifically lists the critical hygiene points in harvesting of bovine heads and cheek meat of cattle. Whilst preparing this opinion, the SSC was informed about a recent outcome of the ongoing UK research, into the pathogenesis of BSE in cattle showing low levels of infectivity in bovine tonsil. In the light of these new data, the TSE/BSE *ad hoc* Group updated its report of 13 December 2001. This update report is attached and it served as the basis for the current update of the SSC opinion on TSE infectivity distribution in ruminant tissues. An English translation of the German report is also attached, for ease of reference (see **Annex 2** of the attached report).

The SSC adopts the following answers to the above questions:

(1) **Tissue infectivity tables applicable for small ruminants.**

Scrapie in small ruminants. There is no new evidence that became available since February 2001 and the SSC's therefore considers that the table attached to its pre-emptive risk assessment of 8-9 February 2001 remains valid. It is annexed as **Table 1** for ease of reference.

BSE in small ruminants. The SSC considers that, pending more experimental data becoming available, it would be prudent on the latest available evidence to adopt tabulations given at **Table 1** as being probably as representative of BSE as scrapie with regard to distribution and level of infectivity in tissues. However, the single and important exception is that lymphoreticular tissues in BSE in sheep should provisionally at least, be considered comparable in their level of infectivity with central nervous system tissues.

(2) **Tissue infectivity tables related to BSE in cattle.** Available data are incomplete and much of the information emanates from a single study of the distribution of infectivity after experimental oral exposure. Available incubation period assay values from the few tissues containing infectivity in experimentally exposed cattle suggests that in most of the infected tissues infectivity is close to the limit of detection of the assay, even in central nervous system. The early results of the re-evaluation of such tissues by bioassay in cattle compliment the mouse data, but such assays will not be completed for at least a further five years. Nevertheless, any further positive results would become available in that period. A tentative summary of available infectivity data for cattle with naturally acquired BSE is given at **Table 2** (Tissues with no infectivity from confirmed cases) and **Table 3** (Preliminary estimates of tissue infectivity after experimental and natural exposure).

(3) **Possible consideration as specified risk material of the entire head of cattle, sheep and goats, including skeletal muscle, tongue and associated innervation.**

Regarding *cattle* affected by or incubating BSE, the SSC considers that there is new evidence from tissue infectivity studies showing that certain head tissues (in

addition to: brain, eyes, dura mater, pituitary gland and skull) could possibly be regarded as SRM at least under certain circumstances. So far results of infectivity bioassays in cattle have supported the view that in the clinical disease stage of BSE, regional lymph nodes, including those of the head have no detectable infectivity. Furthermore, assay results of trigeminal ganglion suggest a low titre of infectivity only in the clinical disease stage, probably secondary to CNS involvement. However, whereas completed results of mouse bioassays of pituitary, cerebro-spinal fluid (CSF), the cranial cervical ganglion, facial nerve, tongue, salivary glands and several lymph nodes of the head from preclinical and clinical stages of experimental BSE in cattle have not revealed infectivity, there is now evidence from cattle-to-cattle transmission studies that the palatine tonsil may contain low levels of infectivity at an early stage of the incubation period and that this may affect the safe consumption of tongue if there is a risk of contamination of this tissue.

There is still no new infectivity data for cattle to suggest that skeletal muscle, tongue or associated nerves should be considered SRM at any age. However, the risk assessment carried out on behalf of the German authorities show that the SSC's initial statement that the "*Exclusion from SRM of bovine tongue and cheek meat remains justified providing contamination by CNS, introduced during slaughter, can be avoided*" may not necessarily be appropriate. That conclusion was reached considering the long list of critical points in the process of slaughtering the animal, the removal, storage and transport of the head and of the harvest of cheek meat.

On the basis of what precedes the SSC considers that:

- (1) the tonsil of a bovine animal of any age should be regarded as posing a risk.
- (2) the tongue of animals certified safe for human consumption does not pose a risk if contamination with CNS and tonsil material is avoided for animals of any age. This may imply that the harvested section of the tongue is shortened [to the "short tongue"], to avoid, by a cautious margin, removal with the tongue of that part of the root of the tongue containing lingual tonsil.
- (3) cheek meat of animals certified safe for human consumption, which is collected as part of a different process (**Annex 2**), does not pose a risk if a

wide range of precautions to avoid cross-contamination is taken. The feasibility of implementation of these precautions under field conditions may however be questioned and would in any case require to be previously verified.

With respect to *sheep*, there is involvement of lymphoid tissue of the head at an early stage of incubation in experimental BSE in sheep, consistent with the view that BSE in sheep has a pathogenesis with respect to tissue distribution of infectivity comparable with natural scrapie. Somatic peripheral nerve trunk infectivity, although categorised as “low” in scrapie, may be widespread in the carcass by the clinical disease stage. If, as seems likely, this results from “centrifugal” spread from the CNS and infectivity can be detected in the CNS in experimental BSE of sheep approximately 40-50% through the incubation period, infectivity may be present in somatic peripheral nerve fibres from this stage. These observations make it difficult to recommend an appropriate lower age limit for the exclusion of any head tissues of sheep if BSE were confirmed or considered likely in a given population.

Furthermore, the practicalities in slaughtering of small ruminants may necessitate removal of the entire head as SRM at all ages. Also, the risk of cross-contamination of tongue with tissues with likely infectivity from early in the incubation of BSE, with or without penetrative stunning, in small ruminants, is considered high.

Consequently, if BSE is considered to be present in sheep, the whole or entire head, including the tongue, of all ages of sheep should be included in the list of SRMs irrespective of slaughterhouse practices, until evidence to the contrary becomes available.

Very limited data are available for goats. The conclusions for sheep are therefore considered to be a reasonable approximation also for goats.

Table 1: Natural scrapie in sheep and goats: classification of tissues by agent titre in Swiss mice and by age, in pre-clinical and clinical cases of Scrapie in Suffolk sheep and in goats¹ (Re-edited but unammended from Annex: Opinion on The Policy of Breeding and Genotyping of Sheep, 22-23 July 1999) (EC 1999)

Infectivity titres*: A = high ($\geq 10^{4.0}$)
 B = medium ($10^{3.2} - 10^{4.0}$)
 C = low ($\leq 10^{3.2}$ or unknown)
 D = undetectable

| | PRE-CLINICAL | | | | CLINICAL | |
|---------------------------------|--------------|--------------------|------|------|----------------|-------|
| Age (months) | ≤ 8 | 10-14 ² | 25 | > 25 | 34-37 | 38-39 |
| Numbers positive / examined | 0/16 | 8/15 | 1/13 | 1/6 | 9/9 | 3/3 |
| Brain | | | | | A | A |
| Brain (medulla) | | D | C | | | |
| Brain (medulla / di-encephalon) | | | C | | | |
| Brain (cortex mid-brain) | | | D | | | |
| Pituitary | | | | | C | B |
| Spinal cord | | | D | | A | A |
| Cerebro-spinal fluid | | | | | C | C |
| Sciatic nerve | | | | | C | C |
| Thymus | D | | D | | C** | C** |
| Thyroid | | | | | D | |
| Spleen | D | B | C | | B | B |
| Tonsil | D | C | B | | B | |
| Lymph node (RP/MP) | D | B | B | | B | B |
| Lymph node (BM) | | D | C | | B | B |
| Lymph node (PS/PF) | D | C | C | | | |
| Lymph node (PF, 1/9 negative) | | | | | B | |
| Lymph node (PS, 2/9 negative) | | | | | B | |
| Lymph node (supra-mammary) | | | D | | C | B |
| Colon-proximal | | B | B | | B | B |
| Colon-distal | | D | D | | C | C |
| Ileum | D | | | | | |
| Ileum-distal | | B | B | | B | |
| Ileum-proximal | | | | | | B |
| Rectum-distal | | | | | B ⁺ | B |
| Pancreas | | | | | C** | |
| Adrenal | | | D | | C | C |
| Nasal mucosa | | | D | | C | C |

¹ After Hadlow et al. (1979, 1980, 1982), Pattison *et al.* (1964, 1972), Groschup et al. (1996). Regarding DRG: see Report.

² Techniques for the determination of infectivity become more and more sensitive. The age range may go below 10 months. In individual cases, tonsil infectivity has been detected in lambs of 16 weeks. Placenta has been placed in Group C, but titres are unknown.

Table 1 (continued): Natural scrapie in sheep and goats: classification of tissues by agent titre in Swiss mice and by age, in pre-clinical and clinical cases of Scrapie in Suffolk sheep and in goats ¹ (Re-edited but unammended from Annex: Opinion on The Policy of Breeding and Genotyping of Sheep, 22-23 July 1999) (EC 1999)

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- A = high ($\geq 10^{4.0}$)
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- C = low ($\leq 10^{3.2}$ or unknown)
- D = undetectable

| | PRE-CLINICAL | | | | CLINICAL | |
|-----------------------------|--------------|--------------------|------|------|-------------------|-------|
| Age (months) | ≤ 8 | 10-14 ³ | 25 | > 25 | 34-37 | 38-39 |
| Numbers positive / examined | 0/16 | 8/15 | 1/13 | 1/6 | 9/9 | 3/3 |
| Bone marrow | | | | | C** | D |
| Liver | | | | | C** | |
| Blood clot | | D | | | D | D |
| Serum | | D | | | | D |
| Salivary glands | | | D | | D | D |
| Saliva | | | | | D | |
| Muscle- skeletal | | | | | D | D |
| Heart | | | | | D | |
| Kidney | | | | | D | D |
| Lung | | | | | D | |
| Ovary | | | | | D | D |
| Uterus | | | | | D | D |
| Placenta | | | | | C*** ^o | |
| Fetus | | | | | D | |
| Mammary gland | | | | | D | D |
| Colostrum | | | | D | | |
| Milk | | | | | | D |
| Semen vesicle | | | | | D | |
| Testis | | | | | D | |
| Faeces | | D | | | | D |

- * = Log₁₀ mouse intracerebral I.D/50 per 30 mg tissues; (titres given as approximate ranges)
- ** = trace or exceptional
- + = Not assayed but high content of lymphoreticular tissue
- ° = negative in other studies
- MP = Mesenteric/portal
- PF = Prefemoral
- CSF = Cerebro-spinalfluid
- PS = Prescapular
- LN = Lymph node
- RP = Retropharyngeal
- BM = Bronchomediastinal

³ Techniques for the determination of infectivity become more and more sensitive. The age range may go below 10 months. In individual cases, tonsil infectivity has been detected in lambs of 16 weeks. Placenta has been placed in Group C, but titres are unknown.

Table 2: Tissues from confirmed cases of BSE in cattle in which no infectivity was detected by bioassay in mice injected both intracerebrally and intraperitoneally (Taken from Kimberlin, 1996)

| | |
|---|--|
| <i>Nervous tissues</i> Cerebrospinal fluid Cauda equina Peripheral nerves : - sciaticus - tibialis - splanchnic | <i>Lymphoreticular tissues*</i> Spleen Tonsil Lymph nodes - prefemoral - mesenteric - retropharyngeal |
| <i>Alimentary tract</i> Oesophagus Reticulum Rumen (pillar) Rumen (oesophageal groove) Omasum Abomasum Proximal small intestine Distal small intestine Proximal colon Distal colon Rectum | <i>Reproductive tissues</i> Testis Prostate Epididymis Seminal vesicle Semen Ovary Uterine caruncle Placental cotyledon Placental fluids : - amniotic fluid - allantoic fluid Udder Milk |
| <i>Other tissues</i> Blood : - buffy coat - clotted - foetal calf - serum Bone marrow Fat (midrum) Heart Kidney | Liver Lung Muscle - semintendinous - diaphragma - longissimus - masseter Pancreas Skin Trachea |

* Tonsil was found positive in the cattle bio-assay – see opinion and attached report.

Table 3: Tentative summary of preliminary estimations* on classification of tissues of cattle according to infectivity after experimental oral or natural exposure to the agent of BSE.

Infectivity titres:**

A = high: $10^{3.0} - 10^{5.0}$ in mouse; $10^{5.7} - 10^{7.7}$ in cattle ***
 B = medium $10^{1.5} - 10^{3.0}$ in mouse; $10^{3.3} - 10^{5.6}$ in cattle ***
 C = low $\leq 10^{1.5}$ in mouse; $\leq 10^{3.2}$ in cattle ***
 D = undetectable
 ? = data not published

| | EXPERIMENTAL | | | | NATURAL |
|--|----------------|----|-------|----------|----------|
| | | | | Clinical | clinical |
| months after exposure | 6-14 | 18 | 32 | 36-40 | - |
| Brain | | | B / C | C | A |
| Retina | | | | | ? |
| Spinal cord | | | C | C | A |
| Dorsal root ganglia | | | C | C | C |
| Trigeminal ganglion | | | | C | |
| Ileum-distal | B / C | C | | C | |
| Palatine tonsil | C [†] | | | | |
| Lymph node (Retropharyngeal) | | | | | D |
| Lymph node (Mesenteric) | | | | | D |
| Lymph node (Popliteal) | | | | | D |
| For the list of tissues in which no detectable infectivity was found: see tables 1 and 2 of this opinion and table 5 and the Annex of the attached report. | | | | | |

*. Refer to the report for further detail

** The classification used is preliminary and arbitrary because of a skewed range of infectivity in cattle with BSE compared to sheep with scrapie. It does not correspond to the Groups or Categories used in Table 1.

*** Values in bold in the table are based on bioassay in cattle.

† Preliminary evidence of estimated titre of $< 10^1$ in cattle



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REPORT ON TSE INFECTIVITY DISTRIBUTION IN RUMINANT TISSUES (STATE OF KNOWLEDGE, DECEMBER 2001)

PREPARED BY THE TSE/BSE *AD HOC* GROUP

Initially finalised at its meeting of 13 December 2001 and updated at its meeting of 10 October 2002 following the submission of (1) a risk assessment by the German Federal Ministry of Consumer Protection, food and Agriculture and (2) new scientific evidence regarding BSE infectivity distribution in tonsils

Rapporteur: Dr.G.A.H.Wells

REPORT ON TSE INFECTIVITY DISTRIBUTION IN RUMINANT TISSUES (STATE OF KNOWLEDGE, OCTOBER 2001)

TABLE OF CONTENTS

| | Page |
|--|------|
| I. Mandate | |
| II TSE infectivity levels in ruminant tissues | |
| II.1. Previous tabulated data | |
| II.2. Context of published data | |
| II.3. Scrapie in sheep : bioassays of sheep tissues after oral or natural exposure to the agent of scrapie by inoculation of mice | |
| II.4. BSE in cattle: bioassay of tissues from cattle experimentally infected with BSE agent and killed sequentially (VLA Pathogenesis study) by inoculation of mice. | |
| II.5. BSE in cattle: Bioassay of cattle tissues by inoculation of cattle | |
| II.6. BSE in sheep: Bioassays of sheep tissues after oral exposure to the agent of BSE by inoculation of mice. | |
| II.7. Conclusions | |
| II.7.1. TSEs in sheep (and goats) | |
| II.7.2. BSE in cattle: | |
| III. The safety of ruminant heads | |
| III.1. Infectivity in relation to incubation period | |
| III.1.1. Bovine | |
| III.1.2. Sheep | |
| III.2. Factors associated with age | |
| III.3. Factors associated with slaughter protocols | |
| III.4. Conclusions | |
| IV. Acknowledgements | |
| V. References | |
| Annex 1 Infectivity titres (bio-assayed in mice) in tissues from Suffolk sheep and goats, at the clinical stage of natural scrapie compared with the titres in tissues from confirmed cases of BSE. | |
| Annex 2: Harvesting of cheek meat of cattle. Specifically: List of critical hygiene points in harvesting of bovine heads and cheek meat of cattle (by Dr L. Ellerbroek and Dr I. Schütt-Abraham | |

I. MANDATE

The Scientific Steering Committee (SSC) was invited:

- (1) To update, on the basis of the most recent scientific data, the sheep tissue infectivity titre table presented in the SSC opinion of 22-23 July 1999 on The Policy of Breeding and Genotyping of Sheep;
- (2) To create a similar table for cattle on the basis of all available scientific evidence;
- (3) To consider whether any new evidence exists since the adoption of its opinion of 9 December 1997 on the listing of Specified Risk Materials which would indicate that the entire head of cattle, sheep and goats, including skeletal muscle, tongue and associated innervation should be considered as specified risk material.

The SSC invited the TSE/BSE *ad hoc* Group to prepare a scientific report that could serve as the basis for preparing an answer to the above question. A first report was finalised by the TSE/BSE *ad hoc* Group at its meeting of 13 December 2001 it served as the basis for an opinion adopted by the SSC at its meeting of 10-11 January 2002.

On 10 May 2002, the Government of the Federal Republic of Germany requested the European Commission to elicit the opinion of the Scientific Steering Committee on a report of 25 April 2002 prepared by the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV), which evaluates the possible risks related to harvesting cheek meat and more specifically lists the critical hygiene points in harvesting of bovine heads and cheek meat of cattle. Whilst preparing this opinion, the SSC was informed about a recent outcome of the ongoing UK cattle pathogenesis research, showing very low levels of infectivity in bovine tonsil. In the light of these new data, the TSE/BSE *ad hoc* Group updated its report of 13 December 2001. This update report is follows hereafter. An English translation of the German report is also attached as **Annex 2**, for ease of reference.

Keywords: Bovine Spongiform Encephalopathy, Transmissible Spongiform Encephalopathy, specified risk material, cattle, small ruminants, sheep, goat, head, tongue, tissue infectivity.

II TSE INFECTIVITY LEVELS IN RUMINANT TISSUES

II.1. PREVIOUS TABULATED DATA

The most recent tabulation of all available data with respect to classification of tissues from clinical cases of scrapie in Suffolk sheep and in goats on the basis of titre of infectivity after assay in mice was given as an annex in the Opinion on The Policy of Breeding and Genotyping of Sheep, Adopted 22-23 July 1999 (EC 1999), and is reproduced here at **Table 1**.

The sheep (Hadlow et al 1982) and sheep and goat data (Hadlow et al 1980) have also been compared previously with preliminary mouse infectivity data on tissues from naturally affected cases of BSE in cattle. This comparison is provided in **Annex**. A list of tissues, from cases of BSE affected cattle, in which no infectivity had at the time of writing been detected by bioassay in mice was also given in Kimberlin (1996) (See **Table 2**). A preliminary table of infectivity categories for tissues from sheep experimentally exposed orally to the BSE agent was given in Annex 3 of the Report attached to the Pre-emptive Risk Assessment should BSE in small ruminants be found under domestic conditions (See **Table 3**).

Table 1: Natural scrapie in sheep and goats: classification of tissues by agent titre in Swiss mice and by age, in pre-clinical and clinical cases of Scrapie in Suffolk sheep and in goats⁴ (Unammended from Annex: Opinion on The Policy of Breeding and Genotyping of Sheep, 22-23 July 1999) (EC 1999)

| Group | Infectivity Titre (approx.range) | PRE-CLINICAL | | | | CLINICAL | |
|-------|-------------------------------------|--|---|--|------------------|--|--|
| | | SHEEP | | | | SHEEP | GOATS |
| | | ≤8 months.(0/16) | 10-14 months(8/15) ⁵ | 25 months(1/13) | > 25 months(1/6) | 34-57 months(9/9) | 38-49 months(3/3) |
| A | HIGH ≥ 4.0 | | | | | Brain Spinal cord | Brain Spinal cord |
| B | MEDIUM 3.2 – 4.0 | | Colon-proximal, Ileum-distal, LN (RP/MP), Spleen | Colon-proximal, Ileum- distal, LN (RP/MP), Tonsil | | Colon-proximal, Ileum-distal, Spleen, Tonsil LN (BM), LN (PF, 1/9 negative), LN (PS, 2/9 negative), LN (PR/MP), (rectum-distal+), | Colon-proximal, Ileum- proximal, LN (BM), LN (RP/MP), LN (s.mammary), Pituitary, (Rectum-distal +), Spleen |
| C | LOW ≤ 3.2 or titre unknown | | LN (PS/PF) Tonsil | Brain (medulla/ diencephalon), LN (BM), LN (PS/PF), Spleen | | Adrenal, Bone marrow**, Colon-distal, CSF, Liver**, LN (s.mammary x2), Nasal mucosa, Pancreas **, Pituitary, Sciatic nerve, Thymus**, Placenta ** ° | Adrenal, Colon-distal, CSF Nasal mucosa, Sciatic nerve, Thymus |
| D | Undetectable | Ileum, LN (PS/PF) LN (RP/MP), Thymus, Tonsil Spleen | Blood clot, brain (medulla), Colon- distal, Faeces, LN (BM), Serum | Adrenal, Brain (cortex mid-brain), Colon-distal, LN (s. mammary), Nasal mucosa, Salivary glands, Spinal cord, Thymus | Colostrum | Blood clot, Fetus, Heart, Kidney, Lung, Mammary gland, Muscle-skeletal, Ovary, Saliva, Salivary gland, Sem. Vesicle, Testis, Thyroid, Uterus | Blood clot, Bone marrow, Faeces, Kidney, Mammary gland, Milk, Muscle- skeletal, Ovary, Salivary gland, Serum, Uterus |

(-/-) (Number positive / number examined)

* = Log₁₀ mouse intracerebral LD/50 per 30 mg tissues

+ = Not assayed but high content of lymphoreticular tissue

° = negative in other studies

** = trace or exceptional

PF = Prefemoral

PS = Prescapular

RP = Retropharyngeal

MP = Mesenteric/portal

CSF = Cerebro-spinalfluid

LN = Lymph node

BM = Bronchomediastinal

⁴ After Hadlow et al. (1979, 1980, 1982), Pattison *et al.* (1964, 1972), Groschup et al. (1996). Regarding DRG: see text.

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|---|--|
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| <i>Alimentary tract</i> Oesophagus Reticulum Rumen (pillar) Rumen (oesophageal groove) Omasum Abomasum Proximal small intestine Distal small intestine Proximal colon Distal colon Rectum | <i>Reproductive tissues</i> Testis Prostate Epididymis Seminal vesicle Semen Ovary Uterine caruncle Placental cotyledon Placental fluids : - amniotic fluid - allantoic fluid Udder Milk |
| <i>Other tissues</i> Blood : - buffy coat - clotted - foetal calf - serum Bone marrow Fat (midrum) Heart Kidney | Liver Lung Muscle - semintendinous - diaphragma - longissimus - masseter Pancreas Skin Trachea |

* Tonsil was found positive in the cattle bio-assay (SEAC, 2002) – see elsewhere in this report.



Table 3: Experimental BSE in sheep: Distribution of infectivity by incubation stage and PrP genotype and stage of incubation (Taken from Annex 3 of the Pre-emptive Risk Assessment should BSE in small ruminants be found under domestic conditions, adopted by the SSC on 8-9 February 2001) (EC 2001) and updated from recent experimental results, see II.6.4 of Report

| Infectivity titre | Pre-clinical | | Clinical | |
|---|--|--|------------------|---------------------------------------|
| | ARR/ARR, ARR/ARQ | ARQ/ARQ | ARR/ARR, ARR/ARQ | ARQ/ARQ |
| High | | | | Brain Spinal cord Spleen |
| Medium | | Spleen Lymph nodes [estimated, not titrated] Tonsil | | Lymph nodes Tonsil |
| Low | | | | |
| PrP-res detected but infectivity not titrated | | Intestine Forestomachs abomasum | | Intestine Forestomachs abomasum |
| Not detectable | Brain, Spinal cord, Spleen, Lymph nodes, Tonsil | | | |

Notes: The summary table is based on the limited research results currently available in this field. Full literature references are provided in the attached report. The table should be used with caution since it relates to experimental, and not natural BSE in sheep, some data are incomplete and some experiments are on-going. Nevertheless it may serve as a guide to the degrees of risk that may exist. The Table should be updated as new results come forward.

No PrP-res has so far been detected in ARQ/ARR or ARR/ARR animals inoculated with BSE up to 2 years post challenge. On the assumption that PrP-res and infectivity are correlated this would suggest that if these animals are infected the titre of infectivity in the years immediately following challenge is lower (or undetectable) in peripheral tissues of these genotypes than in more susceptible genotypes.

II.2. CONTEXT OF PUBLISHED DATA

The concentration of infectious TSE agent in tissue is determined by bioassay usually using endpoint titration or, sometimes, though regarded less accurate, by incubation time assay. The experimental models that have most often been used for such assays are inbred strains of mice. Whilst the most practical bioassay model, mice are likely to provide an underestimate of the concentration of agent in sheep or cattle tissues because of the effect of the species barrier. With few exceptions, previous titration data have been expressed in the form of $\log_{10} \text{ID}_{50}$ units according to Kärber (1931). One mean infective or lethal dose (ID_{50}) is defined as the amount of infectivity that will transmit disease to half of a group of inoculated animals. If 1 ml volumes of successive ten fold dilutions of a specimen are inoculated into a total of 20 mice per dilution group (0.05 ml per animal), one ID_{50} would be present in the dilution that transmits disease to 50% (10/20) of the inoculated animals. Titrations often show transmission rates of about 100%, 50%, and 0% in successive 10-fold dilutions and providing the last, the limiting dilution, is determined, the survivorship can be used to calculate the units of ID_{50} in the original undiluted inoculum volume. This is usually corrected to express the units of ID_{50} /g of the tissue.

It must be stressed that experience of end point titration and incubation period assays in laboratory rodents suggests that the assays are most reproducible when examining infectivity of a strain of agent adapted or, ideally, cloned in the assay species. The inter-laboratory reproducibility of end point titration assays, at least with ME7 scrapie has been demonstrated in C57Bl mice (Taylor *et al.*, 2000). The recent use of mouse titrations of infectivity for cattle and sheep tissues is in contrast to this since the titrations have been conducted across a species barrier on primary inoculation from cattle or sheep.

There are a number of factors which affect the efficiency of infectivity assays. Route(s) of inoculation affect infectivity titre and dose response curves (Kimberlin and Walker, 1978). The volume(s) of inoculum injected also affects the sensitivity of the assay. In practice, the most efficient route(s) of inoculation are selected to perform the assay. In the assays cited in Tables 1 and 2, the calculated limit of detectability of scrapie infectivity by the intracerebral (i.c.) inoculation of mice is approximately $10^{2.0} \log_{10}$ mouse i.c. ID_{50} /g of tissue (Kimberlin 1994) with a volume of inoculum of 30 μL . Clearly, the volume of inoculum that can be injected intracerebrally in a mouse is limited. In the mouse assays of infectivity of tissues from cases of naturally affected cattle with BSE (Fraser and Foster 1994 and H. Fraser, personal communication), shown in Table 3, a combination of i.c. and i.p. injections was used with a total volume of inoculum of 120 μL , giving a limit of detectability of $10^{1.4} \text{ID}_{50}$ /g (Kimberlin 1996).

Fraser *et al* (1992) showed that end point titration of BSE on primary pass to RIII and C57Bl inbred mouse strains gave closely similar values for infectivity.

The relationship between incubation period and titre has been questioned by many authors (see Masel and Jansen 2001, for review), casting doubt on the validity of

estimating titre by incubation period assays. Certain physical and chemical treatments of scrapie affected mouse brain inocula may alter incubation relative to titre, giving a discrepancy between end point titration and incubation period assay of about 10^1 – 10^2 ID₅₀ (Masel and Jansen, 2001). In an analysis of over one hundred scrapie infectivity titrations in mice (McLean and Bostock 2000) a linear rise in mean incubation period with logarithmic decreasing dose was substantiated, but variability in incubation period rose linearly as dose decreased. Thus estimation of titre from dose response curves is less accurate at low doses.

While there are numerous instances of a poor correlation quantitatively between infectivity (depending on how it is measured) and concentration of PrP^{Sc} (Masel and Jansen, 2001), there are also reports demonstrating a relationship between PrP^{Sc} concentration and TSE infectivity (see Lee *et al.* 2001 for review). A “perfect correlation was observed between infectivity and PrP-res detection” (Race *et al.*, 1998) when mouse bioassay (Rocky Mountain Swiss mice) was compared with PrP immunoblotting for assay of brain, spleen, lymph nodes and placenta from scrapie affected Suffolk sheep. However, the nature of this comparison was confined to morbidity data and relative incubation period in the mouse assay (i.e. incubation period was not calibrated against a dose response curve) and presence of PrP-res. Thus in effect the comparison is essentially only qualitative.

Comparisons of the performance of the Bio-Rad version of the CEA Elisa test for rapid detection of PrP^{Sc} with mouse titration data for BSE affected bovine brain has indicated a good correlation, providing prospects for estimations of titre from such rapid test results (Deslys *et al.* 2001). This study was confined to brain tissue and rapid PrP tests are not yet available with suitable methodology for other than central nervous system tissue.

Nevertheless, increasing trend toward the use of PrP^{Sc} detection in preference to the time consuming bioassays as a diagnostic marker means that some examination of the measurement of PrP^{Sc} concentration as a proxy for infectivity should be considered where infectivity data *per se* is lacking. In this way for risk assessment purposes it may be possible to provide better estimates from current data rather than basing assessments purely on historical infectivity data. This has not, however, been pursued in the context of the present report and caution should be exercised in quantitation of PrP^{Sc} as a surrogate for infectivity assays. PrP^{Sc} can indeed indicate the presence of infectivity but in some species transmissions infectivity assays are still much more sensitive. Furthermore, the ratio of amounts of PrP^{Sc} to infectivity may vary. Strain of agent (TSE source), PrP genotype, breed of animal, tissue type and stage in the incubation period may all affect the relative amounts of infectivity to PrP^{Sc}.

The use of transgenic mice with modification to enhance the sensitivity of detection of the donor species infectivity may provide comparative data with assays conducted in conventional mice, but no comparative titration results are, as yet, available for such models (Buschmann *et al.*, 2000).

Titration of infectivity in TSE's have been performed largely on central nervous system tissue, notably brain. Recent data on other tissues is confined to a small number of experiments.

II.3. SCRAPIE IN SHEEP : BIOASSAYS OF SHEEP TISSUES AFTER ORAL OR NATURAL EXPOSURE TO THE AGENT OF SCRAPIE BY INOCULATION OF MICE

There are no recent titrations or incubation period data on tissues of sheep experimentally infected with scrapie via the oral route. Such data on natural cases of scrapie are confined to titres of brain tissue. A pool of 2867 brains of suspect scrapie cases used in a study of the effects of rendering upon the scrapie agent gave a titre of $10^{4.1}$ mouse (i.c. ID₅₀/g of tissue (Taylor *et al.* 1997) compared to the average titre of infectivity in brains of Suffolk sheep clinically affected with scrapie of 10^5 mouse (i.c.) ID₅₀/g (Hadlow and others, 1979). A pool of scrapie affected sheep brains used for the oral exposure of pigs to the scrapie agent gave widely differing infectivity values when titrated in different strains of mice. Titres were $10^{3.7}$ mouse (i.c. + i.p.) ID₅₀/g in IM mice compared to $10^{2.8}$ mouse (i.c. + i.p.) ID₅₀/g in C57BL mice (S.A.C. Hawkins, personal communication).

II.4. BSE IN CATTLE: BIOASSAY OF TISSUES FROM CATTLE EXPERIMENTALLY INFECTED WITH BSE AGENT AND KILLED SEQUENTIALLY (VLA PATHOGENESIS STUDY) BY INOCULATION OF MICE.

The study design has been described previously (Wells *et al.* 1996, Wells *et al.*, 1998). Briefly, forty Friesian/Holstein calves, born in 1991, were assembled from farms with no history of BSE. At four months of age, thirty were each dosed orally with 100g of pooled brain stems from seventy-five cases of BSE. Ten calves received no treatment and served as controls.

Clinical monitoring of cattle was maintained throughout the study to detect the onset of clinical disease.

Starting at six months of age, and then at four month intervals, until 22 months p.i., three challenged calves and one control calf were killed. Thereafter challenged and control cattle were killed at discretionary intervals, with the final kill at 40 months p.i.

Tissues were sampled aseptically for infectivity assays in mice. After each sequential kill, inocula were prepared from 44 tissues, representing principally the lymphoreticular system (LRS), the peripheral nervous system (PNS) and the central nervous system (CNS), alimentary tract, striated muscles and major viscera (see Table 3.1, Wells and others, 1996). All inocula were prepared as ten per cent suspensions in saline, with the inclusion of antibiotics for certain tissues. Single tissue inoculum pools were made from the exposed cattle at each time point. Inocula were similarly prepared but from single tissues of each control animal. Test and control inocula were injected by intracerebral (20µl) and intraperitoneal (100µl) routes into inbred mice for standard qualitative assay of infectivity. Inocula prepared from cattle killed up to 18 months p.i. were injected into RIII mice and/or C57Bl-J6 mice.

Qualitative assays by the i.c. and i.p. inoculation of mice (RIII and/or C57BL) of a large range of tissues from the UK VLA Pathogenesis study of BSE have been completed (Wells *et al.*, 1996, 1998, 1999 and unpublished data). No titration of infectivity in positive tissues has been carried out. For all tissues in which infectivity has not been detected it can be stated that they contain less than $10^{1.4}$ mouse (i.c./i.p.) \log_{10} LD₅₀/g. The results are summarised in **Table 4**.

Prospects for further analysis of the data from tissues in which infectivity has been detected to give an approximation to titre, must rely on survival data, dose and incubation period data for RIII and C57BL mice. The analyses of these data are as yet incomplete, particularly with respect to data on RIII mice (G.A.H. Wells and S.A.C. Hawkins, unpublished). Where data on incubation periods for RIII or C57BL mouse assays on tissues from the Pathogenesis Study of BSE is available, approximations to tissue infectivity titres have been estimated and provided in **Table 4**. These are necessarily provisional since most currently available values are from assays conducted in C57 BL mice, for which only a single experiment dose response curve result is available (on brain and after i.c. inoculation only). More values for incubation periods of RIII infectivity assays, on which titres can be estimated from summated data of a series of dose response curves (after i.c. + i.p. inoculations) will become available in the near future. [GW: *I will attempt to see if more information is now available and provide it at our next meeting*] From the available data it is not possible to estimate more accurately than the very low values of estimated infectivity ($<10^1$ mouse i.c. + i.p. ID₅₀/g) for the majority of the tissues of positive assays in the Pathogenesis study (**Table 4**).

A possible explanation for the very low estimates of infectivity in central nervous system may lie in the relatively early clinical status of cattle killed 32-40 months in the Pathogenesis Study. From a range of titrations conducted on brain from clinical or clinical suspect cases of BSE a wide range of titres have been obtained ($10^{2.9} - 10^{5.2}$ mouse i.c. or i.c + i.p) ID₅₀/g) (Fraser et al 1992, Taylor et al 1994, Kimberlin 1996, G. A. H. Wells and S.A.C. Hawkins, unpublished). It must be emphasised that this variation has to some extent a basis in sampling in that the highest titres were obtained from hind brain from single cases of terminally affected cattle, whereas the lowest titres were obtained from pools of whole brains from clinically suspect cases of BSE (which could contain $\geq 10\%$ negative cases)

Table 4. Summary of Results of Infectivity Assays in mice of Tissues from sequentially killed cattle exposed orally to the BSE agent. (S.A.C.Hawkins & G.A.H.Wells, unpublished)

| Tissue | Infectivity | Estimate of range of titre of infectivity (mouse i.c./i.p. LD ₅₀ /g) relative to incubation period (months) of donor cattle |
|---|-------------|--|
| Neural: | | |
| Brain: frontal cortex, caudal medulla | +, + | [C57BL] $\leq 10^{1.0}$ (32-40m) |
| Pituitary | - | |
| Cerebrospinal fluid | - | [C57BL] $\leq 10^{1.0}$ (32-40m) |
| Dura | N.D. | |
| Spinal cord: C2-C3, T10-T11, L3-L4 | +, +, + | [C57BL] $\leq 10^{1.0}$ (32-40m) |
| Nodose ganglia | - | |
| Dorsal root ganglia: C3-C6, T5-T8 | + | [C57BL] $\leq 10^{1.0}$ (32-40m) |
| Trigeminal ganglia | + | |
| Stellate ganglia | - | [C57BL] $\leq 10^{1.0}$ (32-40m) |
| Sciatic nerve | - | |
| Facial nerve | - | N.D. |
| Phrenic nerve | - | |
| Radial nerve | N.D. | N.D. |
| Semitendinosus muscle | N.D. | |
| Diaphragmatic muscle | N.D. | N.D. |
| Triceps muscle | - | |
| Masseter muscle | N.D. | N.D. |
| Sternocephalicus muscle | - | |
| Longissimus dorsi muscle | - | |
| Alimentary: | | |
| Tongue (dorsum, include mucosa) | - | [R111] $< 10^{0.5} - 10^{1.5}$ (6-14m), $10^{1.2}$ (18m) [C57BL] $< 10^1$ (36-40m) |
| Submandibular salivary gland | - | |
| Parotid salivary gland | - | |
| Cranial esophagus | N.D. | |
| Rumen | - | |
| Omasum | N.D. | |
| Abomasum (pyloric) | - | |
| Duodenum | - | |
| Distal ileum (inc. Peyer's patches) | + | |
| Spiral colon | - | |
| Faeces [‡] | - | |
| Pancreas | - | |
| Liver | - | |
| Lymphoreticular: | | |
| Spleen | - | Note: Tonsil was found positive in the cattle bio-assay (SEAC, 2002) – see elsewhere in this report |
| Thymus (cervical) | - | |
| Tonsil | - | |
| Submandibular lymph node | - | |
| Retropharyngeal lymph node | - | |
| Bronchial-mediastinal lymph node | - | |
| Hepatic lymph node | - | |
| Mesenteric lymph node | - | |
| Prescapular lymph node | - | |
| Popliteal lymph node | - | |
| Other: | | |
| Kidney | - | [C57BL] $< 10^{1.0}$ (38m) |
| Urine [‡] | - | |
| Adrenal | N.D. | |
| Lung (left caudal lobe) | - | |
| Nasal mucosa (midturbinate) | - | |
| Pericardium [‡] | - | |
| Heart (left ventricle/ septum) | - | |
| Mitral valve [‡] | - | |
| Aorta [‡] | - | |
| Blood (buffy coat) | - | |
| Blood (serum) | N.D. | |
| Blood (clot) | N.D. | |
| Bone marrow (sternum) | + | |
| Collagen (Achilles tendon) [‡] | - | |
| Skin [‡] | - | |
| Bone (femoral diaphysis) [‡] | - | |

Key to Table 4:

- + positive
- negative (i.e. $< 10^{1.4}$ mouse (i.c. + i.p.) log₁₀ LD₅₀/g)
- N.D. Not Done (collected and reserved for future study)
- [‡] Selected tissue assays in R111 mice conducted at only two kill time-points (18 and 32 months after exposure).
- * Very low level of infectivity detected only at one time point (38 months after exposure) which is within the range of onset of clinical signs (end of incubation period) for cattle exposed in the study (Wells *et al* 1999)

II.5. BSE IN CATTLE: BIOASSAY OF CATTLE TISSUES BY INOCULATION OF CATTLE

In contrast to the widespread infectivity found in lymphoid tissues of cases of scrapie of sheep, the failure of the mouse bioassay to detect infectivity in tissues outwith the central nervous system of cattle naturally affected with BSE raised the issue of the efficiency of this assay system for the BSE agent. A study was therefore initiated (VLA/CSG SE1821) to provide a measure of the underestimation of the titre of infectivity in tissues across a species barrier in mice and to produce an approximate dose-incubation curve for infectivity of brain from BSE affected cattle by simultaneous titration of a primary inoculum in cattle and in mice. In addition, spleen and lymph node collected from natural cases of BSE were assayed in cattle to provide an order of magnitude estimate of concentration of infectivity in these tissues.

At approximately 4 months of age groups of calves were injected intracerebrally (i.c.) each with a single dilution of inoculum prepared from pooled brain stems from BSE affected cattle using a ten fold dilution range of 10^{-3} to 10^{-8} . Two additional groups of calves were similarly inoculated with a 10^{-1} dilution of a pool of spleen or lymph nodes. All calves were monitored clinically and retained until definite signs of clinical disease developed when they were killed and the brain examined to confirm the morphological phenotype of BSE and the presence of disease specific PrP by immunohistochemistry. A parallel titration in sinc^{s7} (RIII) mice was conducted according to standard mouse end point titration protocols over a dilution range of 10^{-1} to 10^{-6} . Mice were inoculated by the i.c. and intraperitoneal (i.p.) routes simultaneously to maximise the efficiency of the assay.

Brain titres of $10^{3.3}$ mouse (i.c. + i.p.) ID₅₀/g and $10^{6.0}$ cattle (i.c.) ID₅₀/g were established. The resultant value of the underestimation of the infectivity titre of BSE tissue when titrated across a species barrier in mice is therefore a factor of 500 fold (G.A.H.Wells and S.A.C.Hawkins, unpublished data). Expressed as relative titres, 10^0 mouse (i.c./i.p.) LD₅₀/g is equivalent to $10^{2.7}$ cattle (i.c.) LD₅₀/g, or the limit of detection of the mouse bioassay (at approximately $10^{1.4}$ mouse [i.c./i.p.] LD₅₀/g) is equivalent to $10^{4.1}$ cattle [i.c.] LD₅₀/g. Additional assays of selected tissues from the original pathogenesis study by intracerebral inoculation of cattle initially confirmed infectivity only in certain tissues which were already found to be positive by the mouse bioassay. However, recent results (SEAC, 2002) show the presence of low levels of infectivity in bovine tonsil from cattle 10 months after experimental oral exposure to BSE infection. So far only 1 of the group of 5 intracerebrally inoculated recipient cattle has developed disease. SEAC (2002), considered that this finding was unlikely to be an experimental artefact or due to residual infectivity in inoculum. An overall review of the results of additional assays of selected tissues, from the pathogenesis study, by intracerebral inoculation of cattle (as of August 2002) is presented in **Table 5** (G.A.H.Wells and S.A.C.Hawkins, unpublished data).

That the relative degree of insensitivity of the mouse bioassay could explain the apparent absence of widespread LRS infectivity in BSE is not supported by the

results of assays by intracerebral inoculation of cattle with pooled lymph nodes (retropharyngeal, mesenteric and popliteal) or pooled spleens from five terminal clinical cases of BSE. In this study survival data suggested that, if present, the concentration of infectivity in these tissues was, at least, less than one, and possibly less than 0.1 cattle (i.c) LD₅₀/g.

Table 5: Bioassay of tissues from cattle exposed orally to BSE agent (Pathogenesis Study) by intracerebral inoculation of cattle (5 per inoculum group): details of inocula, according to sequential kill point of source cattle, inocula and inoculation dates.

| Inoculum (months p.i.) | Date of inoculation | Survival time ⁴ (months) up to 29/8/02 |
|--|---------------------|--|
| Skeletal muscle ¹ (18m p.i.) | 18.10.96 | 71 |
| Liver (18m p.i.) | 4.11.96 | 71 |
| Kidney (18m p.i.) | 6.11.96 | 71 |
| Distal ileum (18m p.i.) | 7.11.96 | Mean incubation period 24 (5/5)⁵ |
| Skeletal muscle ¹ (32m p.i.) | 11.11.96 | 70 |
| Liver (32m p.i.) | 13.11.96 | 70 |
| Kidney (32m p.i.) | 14.11.96 | 70 |
| Peripheral nerve ² (32m p.i.) | 9.12.96 | 69 |
| Buffy coat (32m p.i.) | 12.12.96 | 69 |
| Caudal medulla/spinal cord (32m p.i.) | 23.2.98 | Mean incubation period 23 (5/5) |
| Distal ileum (32m p.i.) | 25.2.98 | 55 |
| Caudal medulla/spinal cord (22 m p.i.) | 27.2.98 | 55 |
| Thymus (6m p.i.) | 6.4.98 | 53 |
| Distal ileum (10m p.i.) | 8.4.98 | Mean incubation period 22 (5/5) |
| Skin (32m p.i.) | 24.4.98 | 53 |
| Caudal medulla (10m p.i.) | 27.4.98 | 53 |
| Caudal medulla/spinal cord (26m p.i.) | 30.4.98 | 53 |
| Spinal cord (10m p.i.) | 28.5.98 | 52 |
| Spleen (10m p.i.) | 9.7.98 | 50 |
| Tonsil (10m p.i.) | 27.8.98 | 45† (1/5) |
| Thymus (10m p.i.) | 1.9.98 | 49 |
| Kidney (6m p.i.) | 4.9.98 | 49 |
| Liver (6m p.i.) | 21.9.98 | 48 |
| Skeletal muscle (6m p.i.) | 22.9.98 | 48 |
| Regional lymph nodes ³ (6m p.i.) | 24.11.98 | 46 |
| Peripheral nerve ² (6m p.i.) | 26.11.98 | 46 |
| Buffy coat (6m p.i.) | 30.11.98 | 45 |
| Spleen (6m p.i.) | 2.12.98 | 45 |
| Tonsil (6m p.i.) | 3.12.98 | 45 |
| Distal ileum (6m p.i.) | 22.12.98 | Mean incubation period 27 (5/5) |
| Mesenteric lymph nodes (6m p.i.) | 23.12.98 | 45 |
| Caudal medulla (6m p.i.) | 5.1.99 | 44 |
| Spinal cord (6m p.i.) | 7.1.99 | 44 |
| Peripheral nerve ² (18m p.i.) | 11.1.99 | 44 |
| Buffy coat (18m p.i.) | 12.1.99 | 44 |
| Regional lymph nodes (18m p.i.) | 13.1.99 | 44 |
| Salivary gland (18m p.i.) | 19.1.99 | 44 |
| Skin (18m p.i.) | 21.1.99 | 44 |
| Mesenteric lymph nodes (18m p.i.) | 26.1.99 | 44 |
| Spleen (18m p.i.) | 28.1.99 | 43 |
| Tonsil (18m p.i.) | 2.2.99 | 43 |
| Caudal medulla (18m p.i.) | 9.2.99 | 43 |
| Spinal cord (18m p.i.) | 10.2.99 | 43 |
| Skeletal muscle ¹ (26m p.i.) | 11.2.99 | 43 |
| Regional lymph nodes (26m p.i.) | 12.2.99 | 43 |
| Liver (26m p.i.) | 16.2.99 | 43 |

Table 5: Bioassay of tissues from cattle exposed orally to BSE agent (Pathogenesis Study) by intracerebral inoculation of cattle (5 per inoculum group): details of inocula, according to sequential kill point of source cattle, inocula and inoculation dates (continued)

| Inoculum (months p.i.) | Date of inoculation | Survival time ⁴ (months) up to 29/8/01 |
|--|---------------------|---|
| Kidney (26m p.i.) | 18.2.99 | 43 |
| Distal ileum (26m p.i.) | 19.2.99 | 43 |
| Peripheral nerve ² (26m p.i.) | 22.2.99 | 43 |
| Buffy coat (26m p.i.) | 23.2.99 | 43 |
| Salivary gland (26m p.i.) | 25.2.99 | 43 |
| Skin (26m p.i.) | 1.3.99 | 42 |
| Mesenteric lymph nodes (26m p.i.) | 2.3.99 | 42 |
| Spleen (26m p.i.) | 10.3.99 | 42 |
| Tonsil (26m .i.) | 11.3.99 | 42 |
| Caudal medulla (26m p.i.) | 15.3.99 | 42 |
| Spinal cord (26m p.i.) | 16.3.99 | 42 |
| Bone marrow (32m p.i.) | 18.3.99 | 42 |
| Bone marrow (22m p.i.) | 24.3.99 | 42 |
| Bone marrow (36m p.i.) | 29.3.99 | 41 |
| Bone marrow (26m p.i.) | 31.3.99 | 41 |
| Urine (18m p.i.) | 17.8.99 | 37 |
| Nictitating membrane (field case material) | 13.3.00 | 30 |

¹ Pool of semitendinosus, longissimus dorsi and masseter muscles

² Pool of sciatic and radial nerves

³ Pool of prescapular and popliteal lymph nodes

⁴ Survival time of animals remaining in the experiment rounded to nearest whole month (see text)

⁵ No. cattle developing clinical disease/no. inoculated

† Incubation period of single affected recipient. Survival period of remaining 4 recipients in group (at August 29/8/02) is 49 months

From a titration of a pool of BSE affected bovine brain tissue by intracerebral inoculation of cattle a dose/incubation curve has been produced from which it may be possible to obtain an approximation of the titre of an inoculum by reference to incubation period data for that tissue. From the available data to date on the bioassay of Pathogenesis study tissues in cattle, tissues containing infectivity are: distal ileum, 6 m.p.i., 10 m.p.i. and 18 m.p.i. and brain stem/spinal cord, 32 m.p.i. The mean incubation periods for the tissues at these time points, when estimated from the dose/incubation curve for the cattle titration suggest titres of 10^1 - 10^2 (6.m.p.i.), 10^3 (10 m.p.i.), 10^2 - 10^3 (18 m.p.i.) and 10^2 - 10^3 (32 m.p.i.) respectively. This corresponds very approximately to RIII mouse incubation period data in as much as by the mouse bioassay a rising titre (reducing mean incubation) was indicated by the results of distal ileum assay from cattle 6 months and 10 months after exposure and a plateau of incubation period in mice inoculated with distal ileum from cattle 18 months after exposure. The estimated values in certain instances do however show up to a 1 log₁₀ discrepancy between the cattle and mouse infectivity data when estimations are compared

between those calculated on the basis of the cattle i.c. titration dose-response regression curve and those derived from extrapolation from an anticipated 500 fold increased sensitivity over the mouse bioassay. Discrepancies between infectivity estimates by cattle ic titration and extrapolation from the mouse assays fall within the range of experimental error and the values can only be regarded as indicative of general trends.

If one considers the currently available survival times for inoculated cattle in this cattle assay (**Table 5**) it becomes clear that, should there be any infectivity in the remaining tissue groups it would already (as of August 2002) be $<10^1$ cattle i.c. ID₅₀/g for most and considerably lower for some groups. A preliminary summary of infectivity classification for cattle tissues is given in **Table 6**.

II.6. BSE IN SHEEP: BIOASSAYS OF SHEEP TISSUES AFTER ORAL EXPOSURE TO THE AGENT OF BSE BY INOCULATION OF MICE.

II.6.1. The report attached to the *Opinion of the SSC on Specified Risk Materials of Small Ruminants, adopted 13-14 April 2000* (EC 2000) states that from early results of the transmission of BSE to sheep studies (Sheep BSE pathogenesis experiment, carried out by the UK Institute for Animal Health -IAH) some ARQ/ARQ infected sheep have widespread PrP^{Sc} demonstrable in the lymphoreticular system tissues from 16 months after exposure, but there are, as yet, no corresponding bioassay results for infectivity. The report also stresses that this does not exclude finding infectivity or PrP^{Sc} at other (including younger) ages. Additional evidence, not cited in that report (Somerville *et al.*, 1997) demonstrated PrP^{Sc} in spleens of some QQ₁₇₁ Cheviot sheep infected with BSE.

The IAH sheep BSE pathogenesis experiment is ongoing. Immunocytochemical studies of tissues animals succumbing to BSE have been published (Foster *et al.*, 2001). The 7 animals that succumbed to BSE (6 are still alive) all showed PrP^{Sc} immunostaining in CNS and LRS tissues but not elsewhere. While the published results provide information only on clinical cases of experimental BSE in ARQ/ARQ Cheviot sheep (mean incubation period approximately 25 months after exposure to 5g oral dose) it is important to note that tissues from most major organs, including heart, lung, liver or thymus, showed no PrP^{Sc} immunostaining. Minimal staining was seen in glomeruli of the kidney. No evidence of PrP^{Sc} was found in any of the skeletal muscles tested, nor in reproductive tissues or skin.

It is of interest also that of the peripheral nerves examined (vagus, radial, sciatic) only the vagus, which has been proposed by many as implicated in the pathogenesis of scrapie after oral exposures, and not the somatic peripheral nerves, showed PrP^{Sc} immunostaining. Infectivity assays on a range of tissues from these animals are in progress. Studies on animals killed at intermediate times throughout the incubation period are not complete. Preliminary data support the findings of Jeffrey *et al* (2001) which suggest that in some animals evidence of the presence of TSE infectivity (e.g. PrP^{Sc} immunostaining) can be detected in some lymphoid tissues from early on after infection.

II.6.2. Interim updated results of studies by the VLA, UK of the tissue distribution of PrP^{Sc} (Jeffrey *et al*, 2001) and/or infectivity (mouse bioassay) in Romney (ARQ/ARQ) and Suffolk (ARQ/ARQ) sheep orally exposed to the BSE agent (5g affected brain homogenate) (S. Bellworthy, unpublished data) have established the earliest evidence of the presence of agent in tissues as follows:

Romneys (current data on incubation period range: 20-37 months)

| | |
|--|--------------------------|
| - Retropharyngeal lymph nodes (LN) | 4 months after exposure |
| - Peyer's patch | 4 months after exposure |
| - Spleen | 10 months after exposure |
| - Mesenteric LN | 16 months after exposure |
| - Ileocaecal LN | 16 months after exposure |
| - Mediastinal LN | 16 months after exposure |
| - Tonsil | 16 months after exposure |
| - Submandibular LN | 16 months after exposure |
| - Distal ileum (excluding Peyer's patches) | 16 months after exposure |
| - Mesenteric LN | 16 months after exposure |
| - Prescapular LN | 16 months after exposure |
| - Broncho-mediastinal LN's | 16 months after exposure |
| - Brain and spinal cord | 16 months after exposure |
| - Liver (low level of infectivity) | 16 months after exposure |
| - Intestine | 16 months after exposure |
| - Vagus nerve | 16 months after exposure |
| - Forestomachs | 22 months after exposure |
| - Abomasum | 22 months after exposure |
| - Coeliaco-mesenteric ganglion (sympathetic) | 22 months after exposure |

New Zealand Suffolk (current data on incubation period of initial clinical cases: 24 months)

| | | |
|-------------------------------|---|-----|
| - CNS (including spinal cord) | } | 10m |
| - Retropharyngeal LN | } | |
| - Submandibular LN | } | |
| - Prescapular LN | } | |
| - Spleen | } | |
| - Mesenteric LN | } | |
| - Peyer's patch | } | |
| - Ileo-caecal LN | } | |
| - Tonsil | } | |
| - Brain | | 16m |

It must be stressed that there is marked variation in PrP detection results between animals and infectivity bioassay has been conducted on tissue pools from multiple animals. In particular there is no constant pattern of LRS involvement.

This work has also demonstrated PrP^{Sc} immunostaining of neurons in the enteric nervous system (ENS) throughout the alimentary tract (least in forestomachs) in some Romney sheep, but not in sheep that lacked immunostaining in Peyer's patches.

No immunostaining has been detected thus far in thymus, even in clinical cases, nor in somatic peripheral nerve trunks (sciatic, phrenic) or nerve roots of the spinal cord.

There are no new data from this study with regard to possible skeletal muscle infectivity.

Similarly dosed ARQ/ARR (heterozygous for BSE/scrapie susceptibility) Romney sheep are currently approximately four years after dosing and remain healthy.

Sequentially killed animals from this component of the study have not, as yet, shown PrP^{Sc} in any tissues suggesting, at least, that infectivity is extremely low in tissues, certainly up to two years after challenge.

These data suggest that unlike the situation in cattle experimentally infected by the oral route with a relatively large exposure dose of BSE agent, the results in sheep indicate a potentially widespread involvement of lymphoid tissues early in the incubation period at least in ARQ/ARQ scrapie/BSE susceptible sheep. New data are consistent with the previously expressed view that BSE in sheep after oral exposure is pathogenetically closely similar to scrapie, particularly with respect to the tissue distribution of infectivity and/or PrP^{Sc}.

II.6.3. The data published by Houston *et al* (2000) and Hunter *et al* (2002) and show that a high volume blood transfusion from sheep to sheep can transmit a BSE or scrapie illness within the same species and that infectivity can be transmitted from blood taken during the asymptomatic incubation period of the disease of the donor sheep. Little in terms of infectivity data can be drawn from these results and there is insufficient information on the relative efficiencies of routes of infection with BSE in sheep, but one interpretation might be taken from the generally accepted differences between efficiency of routes of inoculation in experimental models. The difference between the efficiency of the oral route and the intracerebral route in cattle is in the range 10^5 to 10^6 (G.A.H. Wells and S.A.C. Hawkins, unpublished). A similar value is frequently cited for the difference in efficiency between such routes in mice. If we assume that the intravenous route is almost as efficient as the intracerebral route, and that this could apply equally to sheep, than in the study cited previously (Jeffrey *et al*, 2001) the oral dose of $10^{4.0} \times 5$ which gave a minimum incubation period of 20 months, the total infectivity contained in 400 ml of blood, producing a similar incubation period, could be as low as 1-10 mouse ID₅₀ units. Notwithstanding discrepancies in making such calculations across sheep breeds this would certainly be undetectable by mouse bioassay.

II.6.4. Although no endpoint titration was conducted, incubation period data from primary transmission of infection from brain and spleen of sheep (Cheviot ARQ) infected intracerebrally or orally with BSE agent showed comparable incubation periods in each tissue (Foster *et al.*, 1996). These incubation periods were shorter than those obtained from the original primary transmissions of cattle BSE agent to mice (Fraser *et al.*, 1992) which gave endpoint titration results of at least $10^{5.1}$ (i.c.) LD₅₀/g. Experiments to compare the effects of i.c. and i.p. routes or their combination on incubation period in RIII mice (Bruce *et al.*, 1994) have shown slightly increased efficiency of detection of BSE infection (from cattle) with the combined route. It might be concluded, therefore, that the titre of infectivity in

the BSE affected sheep brain and spleen tested by Foster *et al* (1996) was of the order of 10^5 i.c./i.p. LD₅₀/g. Caution has been urged with regard to interpretation of incubation period assays in different tissues/organs since it has been shown that on a single pass of 263K hamster scrapie there was modification of the dose-response relationship for spleen compared to brain (Robinson *et al.*, 1990).

There are no titration data on tissues from sheep experimentally infected with BSE agent.

Mouse bioassay of tissues from the VLA study of oral exposure of Romney and Suffolk sheep to BSE agent (Jeffrey *et al* 2001) are incomplete but for some tissues of exposed Romney (ARQ/ARQ sheep) there is sufficient data on incubation period (S.Bellworthy, personal communication) to attempt approximations of titres of infectivity from RIII mouse dose response curves.

By 16 months after exposure (5g dose of $10^{4.0}$ mouse (i.c. + i.p.) ID₅₀/g) it appears that spleen is approaching a titre of approximately $10^{2.8}$ mouse (i.c. + i.p.) ID₅₀/g, lower at 10 months after exposure and increasing thereafter (data incomplete). Other lymphoid tissues at 16 months after exposure are probably $10^{1.0}$ but increasing thereafter and at 22 months after exposure (still preclinical) central nervous system infectivity is $\geq 10^3$.

No data are available as yet from clinically affected sheep (incubation periods 20-28 months (Jeffrey *et al* 2001).

The Annex 3 of the Report: Pre-emptive Risk Assessment Should BSE in Small Ruminants be found under domestic conditions, adopted 8-9 February 2001 (EC 2001a), which is based on results of this study is, therefore, still applicable with regard to classification of tissue infectivity for Romney (ARQ/ARQ) sheep experimentally exposed to the BSE agent (Table 3).

II.6.5. In view of this apparent close similarity in the distribution of infection in tissue between experimental BSE in sheep and natural scrapie it would seem that further guidance on the probability and possible levels of infectivity in different tissues should be drawn from previous tabulations of scrapie infectivity in tissues of small ruminants (see Table 1 and Annex 1).

II.7. CONCLUSIONS

II.7.1. TSES IN SHEEP (AND GOATS)

Scrapie in small (sheep) ruminants

There are no new data from which to update the Table 1 and the Annex 1 for infectivity of tissues of sheep for scrapie. These tables remain therefore valid as far as scrapie infectivity distribution is concerned.

BSE in small (sheep) ruminants

Recent data which would enable updating of sheep tissue infectivity titre tables for infection with the scrapie agent and for infection with the BSE agent are extremely limited. With respect to sheep experimentally exposed to the BSE agent interpretation of data set out above would suggest that infectivity titres in brain and spleen during the clinical disease phase may be comparable. Thus for BSE the possibility has to be considered that spleen (and possibly other lymphoreticular system tissues) may have to be regarded, together with CNS tissues, as containing a High level of infectivity. This is in contrast to previous data (Tables 1 and 2) in which spleen of sheep with scrapie has been assigned Medium infectivity. This clearly has implications for consideration of SRM for sheep where there is a probability of occurrence of BSE in sheep. This accepted, there are no new data from which to update the Tables 1 and 2 for infectivity of tissues of sheep for scrapie or BSE.

With respect to BSE in sheep, it would be prudent on the latest available evidence to adopt tabulations given at **Table 1** and the **Annex 1** as being probably as representative of BSE as scrapie with regard to distribution and level of infectivity in tissues. The single and important exception is that lymphoreticular tissues in BSE in sheep should provisionally at least, be considered comparable in their level of infectivity with central nervous system tissues.

II.7.2. BSE IN CATTLE:

A basis for producing cattle tissue infectivity tables for infection with BSE is emerging but the data are incomplete and much of the information emanates from a single study of the distribution of infectivity after experimental oral exposure. Available incubation period assay values from the few tissues containing infectivity in experimentally exposed cattle suggests that in most of the infected tissues infectivity is close to the limit of detection of the assay, even in central nervous system (**Table 4**). The current results of the re-evaluation of such tissues by bioassay in cattle (**Table 5**) compliment the mouse data. They show that low levels of infectivity (below those detectable by the mouse bioassay⁶) may be present in the palatine tonsil, but, to date not in any of the other tissues which gave negative results on mouse bioassay. The currently still ongoing such assays will not be completed for at least a further four years. Nevertheless, any further positive results would become available in that period. A tentative summary of available infectivity data for cattle with BSE is given at **Table 6**.

⁶ The cattle/mouse species barrier is estimated between 500 to 1000; a lack of detection of infectivity by the mouse bioassay may leave infectivity close to 1 oral infectious dose.

Table 6: Tentative summary of preliminary estimations¹ on classification of tissues of cattle according to infectivity after experimental oral or natural exposure to the agent of BSE.

| Infectivity titre ² (approx. range) | | Experimental | | | Natural (Clinical) |
|---|---|---|---|---|---|
| Mouse | Cattle ³ | Preclinical (months after exposure) | | Clinical (months after exposure) | |
| | | (6-14) | (18) | (32) | (36-40) |
| High (10 ^{3.0} -10 ^{5.0}) | High (10 ^{5.7} -10 ^{7.7}) | | | | Brain Spinal cord ?Retina (data not published) |
| Medium (10 ^{1.5} -10 ^{3.0}) | Medium (10 ^{3.3} -10 ^{5.6}) | Distal ileum (10 months) | Brain | | |
| Low (≤10 ^{1.5}) | Low (≤10 ^{3.2}) | Distal ileum Distal ileum (6 months) Palatine tonsil (10 months)† | Brain Spinal cord Dorsal root ganglia | Brain Spinal cord Dorsal root ganglia Trigeminal ganglion Distal ileum Bone marrow (38 months) | |
| Undetectable ?($<10^{1.0}$) | Undetectable ?($<10^0$) | For list of tissues see Tables 1, 5 & Annex 1 | | | Retropharyngeal LN Mesenteric LN Popliteal LN For remaining tissues tested see Table 2 and associated references |

¹. Refer to **Tables 1, 5** and **Annex 1** for further detail

². The classification used is preliminary and arbitrary because of a skewed range of infectivity in cattle with BSE compared to sheep with scrapie. It does not correspond to the Groups or Categories used in **Table 1** and **Annex 1**. Ranges of comparative values between mice and cattle are based on extrapolation from an anticipated 500 fold increased sensitivity over the mouse bioassay. They are an overestimate according to the cattle i.c. titration dose- response regression curve.

³. Values in bold in the table are based on bioassay in cattle.

† Based on cattle i.c. dose-response curve, infectivity is < 10 cattle i.c. ID₅₀/g



III. THE SAFETY OF RUMINANT HEADS

Note: Particular note is drawn to previous definitions used in Opinions and Reports for the head and its anatomical parts. For the purpose of the current report, "head" and "entire head" are considered the same and include the whole head, including the tongue. The term "skull" in the bovine context is the head excluding cheek meat (Masseter muscle) and tongue. In small ruminants the term "skull" is the head, excluding skin and tongue.

III.1. INFECTIVITY IN RELATION TO INCUBATION PERIOD

III.1.1. Bovine

In relation to the head in cattle with BSE, infectivity is consistently detected in the central nervous system (CNS) in the clinical disease, both in natural and experimental cases. In the experimental disease in cattle infectivity is detected in the CNS prior to the onset of clinical signs. But, the Pathogenesis Study does not provide interpretable data on the relationship between the earliest detectable infectivity in CNS (or any other tissue) and incubation period after experimental oral infection of cattle with the agent of BSE. In naturally occurring BSE, the age at which brain material may contain infectivity is unknown and it is not possible to predict when a case of BSE will show infectivity in the CNS. In the experimental study of BSE in cattle after oral exposure, in which the lower limit of the incubation period range was 35 months, evidence of infectivity [by conventional mouse bioassay] in the CNS was detected at 32 months, but not at 26 months after dosing (Wells *et al.*, 1998). However, these two observations, of clinical onset and tissue infectivity, cannot be compared directly since (given the sequential kill protocol of the study) the incubation period range of all animals in the study cannot be determined. A preliminary estimate from dose response data of cattle infected orally with a dose of BSE infectivity closely similar to that administered to induce disease in the Pathogenesis Study (G. A. H. Wells, unpublished data) suggests a mean incubation of almost 45 months (range 33-55 months). Because there is no direct experimental data relating infectivity of tissues to incubation period in BSE there is no equation that might be applicable to calculate the initial time of detectability of tissue infectivity in relation to incubation of the natural disease. However, in certain experimental mouse models of scrapie, after peripheral routes of exposure, a constant relationship can be shown between the initial detection of infectivity in CNS and incubation. Within the range of models examined, infectivity was detectable at approximately 54% of the incubation period (Kimberlin and Walker 1988; Kimberlin and Walker 1989). It is not known if such a constant relationship might be applicable to BSE of cattle, but data from naturally occurring sheep scrapie where the approximate incubation period is apparent a similar value of 50% has been suggested (Opinion on SRM of Small Ruminants Adopted 13-14 April 2000). Based therefore, on the overall knowledge gained from natural incidents of TSEs in animals, and on available data, it seems not unreasonable to accept that infectivity may be first *detectable* in the CNS in natural BSE well in advance of clinical onset. This might be as little as 3 months before clinical signs, by conventional mouse bioassay, but

theoretically at least, it could be 30 months, in an animal with an average estimated field case incubation of 60 months. BSE infectivity has been assayed in mice and cattle, providing evidence for a cattle-to-mouse species barrier of about 500 fold ($10^{2.7}$) (G. A. H. Wells, unpublished data). As the cattle-to-human species barrier is yet unknown (E.C., 1999), no calculation of infectivity risk for man from an estimated onset of detectable infectivity in cattle CNS can be made.

As indicated earlier, infectivity in trigeminal ganglia (anatomically located within the base of the skull) in experimentally induced BSE has been detected only in the clinical disease stage and is probably secondary to replication of agent in CNS.

However, the recent finding (SEAC, 2002) of BSE in 1 of the group of 5 cattle intracerebrally inoculated with palatine tonsil, from donor cattle 10 months after experimental oral exposure to BSE infection, with an incubation period of 45 months, suggests the possibility that BSE infectivity may be present in tonsils in natural cases of BSE from a young age, albeit probably at low levels.

III.1.2. Sheep

There is little new information as yet, but from the VLA's experimental study of BSE in sheep (exposed to a relatively large dose of 5g of infective brain tissue), it appears that after this dose, involvement of the lymph nodes of the head (retropharyngeal), can be as early as 17% (4 months in the specific study) of the incubation period, and CNS involvement may occur from 40-66% (10-16 months in the specific study) of the incubation period. Clearly, with a range of much lower exposures in field situations that might be anticipated in endemic BSE in sheep and possibly different susceptible PrP genotypes in sheep, there may well be proportionally longer incubation periods and correspondingly later involvement of the CNS. However, it must be considered that dissemination of agent to widespread lymphoid sites may be a relatively constant early event in incubation of scrapie and BSE in sheep but could be influenced by their genotype.

III. 2. FACTORS ASSOCIATED WITH AGE

Age-cut-off limits for the skull, central nervous system, eyes and tonsils for bovine, ovine and caprine animals below which age the named tissue is not considered a risk need to be determined on a case-by-case basis which takes into account the criteria of animal species, infectivity in relation to incubation period, factors associated with slaughter protocols and geographical risk level of the source country or region.

Cattle:

It has been previously established that the incidence of clinical disease occurrence in cattle below 30 months of age is approximately 0.05%. Experimental data also suggests that after oral exposure of calves to BSE infection, doses of the order of 100g of high titre brain material are required to give an incubation period range with a minimum of approximately 30 months (G. A. H. Wells and S. A. C. Hawkins, unpublished data).

So far, results of infectivity bioassays in cattle have supported the view that in the clinical disease stage of BSE, regional lymph nodes, including those of the head have no detectable infectivity. Furthermore, assay results of trigeminal ganglion suggest a low titre of infectivity only in the clinical disease stage, probably secondary to CNS involvement. However, whereas completed results of mouse bioassays of pituitary, cerebro-spinal fluid (CSF), the cranial cervical ganglion, facial nerve, tongue, salivary glands and several lymph nodes of the head from preclinical and clinical stages of experimental BSE in cattle have not revealed infectivity, there is now evidence from cattle-to-cattle transmission studies that the palatine tonsil may contain very low levels of infectivity at an early stage of the incubation period and that this may affect the safe consumption of tongue if there is a risk of contamination of this tissue.

Sheep and goats:

The absence of evidence of naturally occurring cases of BSE in sheep or goats and the preliminary nature of information on the pathogenesis of experimentally induced BSE in sheep prevent clear inferences regarding age factors and the relative infectivity of head tissues. It must be acknowledged that natural exposures to BSE agent via feed or through endemic infection of sheep would probably result in a mean incubation period much like that of naturally occurring scrapie and greater than those resulting from the experimental oral exposures to BSE infection for which there is some data (Foster et al., 1993, and above Bellworthy, personal communication). However, the interactions of dose and host genetics, constituting the variables of effective exposure, do not as yet allow the sort of assessments that have been made in the case of cattle with BSE. Because of this uncertainty and the potential for the involvement of lymphoid tissues of the head at an early stage of incubation in sheep with BSE, there is no basis on which to recommend an age cut-off for the small ruminant head SRM's were BSE to be confirmed in small ruminants. Clearly, this needs also to be considered in relation to the geographical risk of BSE occurring in sheep and, dependent on possible grading of risk, an age cut-off could be applied, as suggested previously [*Opinion and Report from the Working Group: Specified Risk Materials of Small Ruminants, Opinion adopted 13-14 April 2000*] (EC 2000), particularly with respect to certain unprocessed meat products, such as MRM and/or offals (presumed tongue) derived from the head.

III.3. FACTORS ASSOCIATED WITH SLAUGHTER PROTOCOLS

This aspect is discussed in detail in the *Scientific Opinion and Report on Stunning methods and TSE risks* adopted by the SSC on 10-11 January 2002 (E.C., 2002).

Bovine skull:

The definition of bovine skull (entire head less cheek meat and the tongue) and the related non categorisation of bovine tongue as SRM (see above Table 2) may not anymore remain appropriate in relation to certain slaughter procedures. The regulations currently allow removal of tongue provided it is not contaminated

(and can be removed within the confines of the abattoir and before contact with heads from other animals might occur). This would remain a reasonable and practical procedure only if contamination either by CNS material or by tonsil tissue is excluded. The tongue could indeed be at risk from cross contamination with CNS material as a result of leakage from the foramen magnum (and notably from the stun hole if a penetrative method of stunning is used) or cross contamination with tonsil as a result of any method of removal of the tongue that did not ensure careful separation of the tongue from all tonsillar tissues.

Furthermore, head meat under hygiene regulations must be removed in a cutting plant designed for the purpose. The movement of large numbers of heads which are often in contact with each other, from an abattoir to the plant increases the risk of cross contamination. The risk is increased when any penetrative stunning method is used (in the same order of risk as is specified in the report) but is not zero if penetrative stunning is not used because CNS material can still leak from the foramen magnum. It is noted also that all visible nervous and lymphatic tissue must be removed before sale to the consumer and that these tissues (lymph nodes and peripheral nerves) have not revealed detectable infectivity in cattle with natural or experimental BSE.

Thus, there are circumstances where it could be prudent to include the tongue (the entire head) from cattle as SRM. This could be subject to exclusions on the basis of the use of a non-penetrative stunning method, on an age basis and in relation to the status of the BSE epidemic of a particular country. That is, where evidence can be provided of a declining epidemic and all the necessary measures are consistently enforced (see below), because the incidence of disease (and thereby infection) is low and becoming lower with time in younger animals.

Under normal abattoir procedures there is no contact between gut tissues (the only other tissue known to contain infectivity during the incubation period of experimentally induced BSE) and the head.

However, while there are still no new infectivity data from assay of tissues in cattle to suggest that skeletal muscle, tongue or associated nerves should be considered SRM at any age, the hazard identification and risk assessment carried out on behalf of the German authorities (BvGG, 2002) show that the SSC's statement of 10-11 January 2002 that the "*Exclusion from SRM of bovine tongue and cheek meat remains justified providing contamination by CNS, introduced during slaughter, can be avoided*" may not necessarily be appropriate considering the long list of critical points in the process of slaughtering the animal, the removal, storage and transport of the head and harvesting the tongue and cheek meat. The safe harvesting of head tissues would require strict and complex procedures which may not always be realistic under field conditions and which would require major efforts in terms of supervision and control.

Small ruminant skulls:

The classification of skull as SRM in small ruminants (the head excluding skin and tongue) also necessarily excludes the tongue from the SRM list but because of

practicalities of slaughtering it has been suggested that the entire head of small ruminants may be required to be included as SRM at all ages. This would be particularly so in a situation where BSE has been confirmed or is considerably likely to have occurred in a sheep population.

Cross contamination of tongue with CNS from penetrative stunning or from the foramen magnum decapitation is more likely in sheep than in cattle because of skinning of the head. Furthermore, if the CNS is infective then it is highly likely that all lymph nodes of the head, tonsils and possibly peripheral nerves will also contain infectivity.

Avoidance of penetrative stunning only marginally reduces the contamination risk”.

III.4. CONCLUSIONS

There is preliminary new evidence from studies of BSE tissue infectivity by assay of tissues in cattle suggesting a need to review the head tissues of bovine animals which are currently designated as SRM. Concerning the bovine head, Commission Regulation (EC) Number 270/2002 of February 14, 2002 designates the skull including brain⁷ and eyes, dura mater, pituitary gland and the tonsils of animals over 12 months of age, as SRM. For the UK and Portugal (GBR IV) the Regulation designates the entire head (including brain, eyes, trigeminal ganglia and tonsils), excluding tongue, of animals over 6 months of age, as SRM. So far, results of infectivity bioassays in cattle have supported the view that in the clinical disease stage of BSE, regional lymph nodes, including those of the head have no detectable infectivity. Furthermore, mouse assay results of trigeminal ganglion suggest a low titre of infectivity only in the clinical disease stage, probably secondary to CNS involvement. However, whereas completed results of mouse bioassays of pituitary, cerebro-spinal fluid (CSF), the cranial cervical ganglion, facial nerve, tongue, salivary glands and several lymph nodes of the head from preclinical and clinical stages of experimental BSE in cattle have not revealed infectivity, there is now evidence from cattle-to-cattle transmission studies that the palatine tonsil may contain very low levels of infectivity and that this may be present at an early stage of the incubation period. This may affect the safe consumption of tongue if there is a risk of contamination of this tissue. It also implies that tonsil be regarded as SRM from animals of any age.

Furthermore, the SSC's statement of 10-11 January 2002 that the “*Exclusion from SRM of bovine tongue and cheek meat remains justified providing contamination by CNS, introduced during slaughter, can be avoided*” may not necessarily be appropriate anymore considering the long list of critical points in the process of slaughtering the animal, the removal, storage and transport of the head and harvesting the cheek meat. The safe harvesting would require strict and complex procedures which may not always be realistic under field conditions and which would require major efforts in terms of supervision and control.

⁷ The TSE/BSE *ad hoc* Group considers in this respect the brain and connected tissues as the central nervous system in the skull

The TSE/BSE *ad hoc* Group therefore considers that:

- (1) the tonsil of a bovine animal of any age should be regarded as posing a risk.
- (2) the tongue of animals certified safe for human consumption does indeed not pose a risk if contamination with CNS and tonsil material is avoided for animals of any age, but this would imply that the harvested section of the tongue is shortened [to the "short tongue"] to avoid, by a cautious margin, removal with the tongue of that part of the root of the tongue containing lingual tonsil.
- (3) cheek meat of animals certified safe for human consumption, which is collected as part of a different process, does not pose a risk if a wide range of precautions to avoid cross-contamination is taken. The feasibility of implementation of these precautions under field conditions may be questioned and would require to be previously verified.

The other head SRMs if a BSE risk exists remain appropriate for bovines.

With respect to *sheep*, there is involvement of lymphoid tissue of the head at a relatively early stage of incubation in experimental BSE in sheep, consistent with the view that BSE in sheep has a pathogenesis with respect to tissue distribution of infectivity comparable with natural scrapie. Somatic peripheral nerve trunk infectivity, although categorised as "low" in scrapie, may be widespread in the carcase by the clinical disease stage. If, as seems likely, this results from "centrifugal" spread from the CNS and infectivity can be detected in the CNS in experimental BSE of sheep approximately 40-50% through the incubation period, infectivity may be present in somatic peripheral nerve fibres from this stage. These observations make it difficult to recommend an appropriate lower age limit for the exclusion of any head tissues of sheep if BSE were confirmed or considered likely in a given population also because of a possible influence on incubation and tissue distribution by the genotype of the sheep. Furthermore, as stated previously, the practicalities in slaughtering of small ruminants may also necessitate removal of the entire head as SRM at all ages.

Also, the risk of cross-contamination of tongue with tissues with likely infectivity from early in the incubation of BSE, with or without penetrative stunning, in small ruminants, is considered high.

Consequently, if BSE is considered to occur in sheep, the whole or entire head, including the tongue, of all ages of sheep might have to be included in SRM irrespective of slaughterhouse practices. Possible exception to this would require additional risk assessment specifically for the occurrence of endemic BSE in sheep and the application of a geographic BSE (sheep) risk assessment.

IV. ACKNOWLEDGMENTS

The SSC wishes to thank Dr.G.Wells, rapporteur of the 2 detailed reports that served as the basis for the current report and of its update of 7-8 November 2002.

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Annex 1: Infectivity titres (bio-assayed in mice) in tissues from up to 9 Suffolk sheep (34-57 months old) and up to 3 goats (38-49 months old), at the clinical stage of natural scrapie compared with the titres in tissues from 1 or more confirmed cases of BSE (Re-edited but unammended from Kimberlin 1994)

| Tissues | Titre (mean \pm SEM of (n) samples) ^a | | | | Titre ^a |
|---------------------|--|------|----------------|------|--------------------|
| | Scrapie, sheep | | Scrapie, goats | | BSE, cattle |
| Category I | | | | | |
| Brain | 5.6 \pm 0.2 | (51) | 6.5 \pm 0.2 | (18) | 5.3 |
| Spinal cord | 5.4 \pm 0.3 | (9) | 6.1 \pm 0.2 | (6) | +ve |
| Category II | | | | | |
| Ileum | 4.7 \pm 0.1 | (9) | 4.6 \pm 0.3 | (3) | <2.0 |
| Lymph nodes | 4.2 \pm 0.1 | (15) | 4.8 \pm 0.1 | (3) | <2.0 |
| Proximal colon | 4.5 \pm 0.2 | (3) | 4.7 \pm 0.2 | (3) | <2.0 |
| Spleen | 4.5 \pm 0.3 | (3) | 4.5 \pm 0.1 | (3) | <2.0 |
| Tonsil | 4.2 \pm 0.4 | (9) | 5.1 \pm 0.1 | (3) | <2.0 |
| Category III | | | | | |
| Sciatic nerve | 3.1 \pm 0.3 | (9) | 3.6 \pm 0.3 | (3) | <2.0 |
| Distal colon | <2.7 \pm 0.2 | (9) | 3.3 \pm 0.5 | (3) | <2.0 |
| Thymus | 2.2 \pm 0.2 | (9) | <2.3 \pm 0.2 | (3) | not done |
| Bone marrow | <2.0 \pm 0.1 | (9) | <2.0 | (3) | <2.0 |
| Liver | <2.0 \pm 0.1 | (9) | -- | | <2.0 |
| Lung | <2 | (1) | <2.1 \pm 0.1 | (2) | <2.0 |
| Pancreas | <2.1 \pm 0.1 | (1) | -- | | <2.0 |
| Category IV | | | | | |
| Blood clot | <1.0 | (9) | <1.0 | (3) | <1.0 |
| Heart muscle | <2.0 | (9) | -- | | <2.0 |
| Kidney | <2.0 | (9) | <2.0 | (3) | <2.0 |
| Mammary gland | <2.0 | (7) | <2.0 | (3) | <2.0 |
| Milk* | -- | | <1.0 | (3) | not done* |
| Serum | -- | | <1.0 | (3) | <1.0 |
| Skeletal muscle | <2.0 | (9) | <2.0 | (1) | <2.0 |
| Testis | <2.0 | (1) | -- | | <2.0 |

The data are taken from the following sources: sheep scrapie, Hadlow *et al* (1982); goat scrapie, Hadlow *et al* (1980); BSE, Fraser *et al* (1992), Fraser & Foster (1994), and Kimberlin (1994). The classification of tissues is according to the CJD Prion Guidelines (EC, 1991). The Table is from Kimberlin (1994) and has been reproduced previously as Table 3 in the SS Opinion of 9 December 1997 providing a *Listing of Specified Risk Materials (re-edited 22 January 1998)* and in *SEAC Report 1994*, (Table 5.2 Amended). The only positive bovine tissue (brain) for which a titre is quoted, is from Fraser *et al* (1992). The remaining tabulation for negative tissues of cattle provides the cut off of sensitivity of the assay according to standard calculation of the minimum detectable titre taking into consideration volume of inoculum used. The <1 and <2 entries quoted in the table are in the original paper. The <1 values may relate to the possibility that inoculum used for blood clot and serum was undiluted, but this is not stated in the source paper of the bioassay of tissues from clinical cases of BSE (Fraser and Foster 1994), or (Kimberlin 1994).

^aTitres are expressed as arithmetic means of log₁₀ mouse i/c. LD 50/g or ml of tissue (+ve > 2.0).

+ve = transmission positive but not titrated

NOTE: None of the bovine tissues in categories II and III and no tissues in Category IV had any detectable infectivity. The values shown are minima based on the limits of detectability of the bioassay in mince (calculated for 30µl of inoculum injected intracerebrally).

* Data on the negative results of bioassay of milk from cattle with BSE were not available in Kimberlin (1994). Subsequently, negative results of bioassay in mice were published and cited by Kimberlin (1996), see Table 2 of Report.

Annex 2:

HARVESTING OF CHEEK MEAT OF CATTLE

SPECIFICALLY: LIST OF CRITICAL HYGIENE POINTS IN HARVESTING OF
BOVINE HEADS AND CHEEK MEAT OF CATTLE

Report submitted on 25 April 2002 by the German federal Institute for Consumer Health Protection and Veterinary Medicine (BvGG) to the Government of the federal Republic of Germany

Rapporteurs:

Dr L. Ellerbroek

Dr J. Schütt-Abraham

As instructed, we herewith deliver the following opinion, in agreement with the Federal Institute for Meat Research (BfL 7)

In the framework of talks between the Federation and the *Länder* on 15 April 2002 at the BMVEL, we discussed how to proceed with the transposition of Regulation (EC) 270/2002 of 14 February 2002 amending Regulation (EC) 999/2001. The amended Regulation provides that the entire head (without tongue) must be removed in the slaughterhouse as specified risk material, unless a Member State permits the harvesting of cheek meat or tongues in cutting plants specifically authorised for this purpose.

Considerable quantities of meat are currently harvested from bovine heads. According to the Federal Statistical Office, a total of 4 114 093 cattle and 485 43 calves were slaughtered in Germany in 2001. According to estimates furnished by the economic operators concerned, half of the harvested bovine heads come from animals aged over 30 months, mainly cows. Depending on age and sex, up to 6 kg meat can be harvested from a bovine head. This includes approximately 2 kg (internal and external) cheek meat, approximately 2 kg neck meat, approximately 1 kg skull meat (i.e. meat attached to the skull), approximately 200 g temple meat and approximately 1 kg snout meat; at present this meat is mainly used in the feedstuffs industry (as feedingstuffs for animals not intended for human consumption).

Since the derogation in Regulation (EC) 270/2002 applies only to cheek meat of cattle and the harvesting of neck meat, skull meat, temple meat and snout meat will not be permissible in future even under the derogation, the amount of meat harvestable from bovine heads will be reduced to a maximum of 2 kg per head. However, in individual cases it will be impossible to meet compliance with the restriction prescribing the harvesting of cheek meat only.

In our report of 10 January 2001 (BvGG BgVV 5222-04-133846) we already indicated a number of critical points where meat can be contaminated with the BSE agent during slaughter. Bearing in mind the usual cattle slaughtering practices in Germany, contamination with BSE agent cannot be ruled out, particularly when harvesting and processing bovine heads.

In order to prevent contamination in general and contamination with BSE risk material in particular, we consider that comprehensive precautionary measures are of the essence. Notably, care should be taken to avoid contamination of meat with fluids containing brain tissue residue.

The Scientific Steering Committee (SSC) came to the same conclusion and stated in its opinion of 9 December 1997 that the entire head excluding the tongue is to be considered as “organs” with high BSE-infectivity. This is because of the possible risk of contamination with the TSE agent when harvesting heads and subsequent handling. During usual slaughtering practice, contamination of the meat with tissue which may have a very high degree of TSE-infectivity in the case of infected animals is unavoidable. When the head is removed, fluid spills out through the foramen magnum and the spinal cord channel; besides, spinal cord tissue is cut. In addition, in the case of captive bolt stunning, blood containing brain material may spill through hole in the forehead, contaminate the skin of the scalp and enter the meat during subsequent handling of the head.

However, depending on the stunning practice and slaughtering technique, the extent of this external contamination with blood, fluid and brain material can vary. In individual cases, brain material may even gush out of the opening as a result of the way stunning is performed. During subsequent skinning of the head this contamination inevitably reaches the meat and may subsequently spread over the entire surface of the head.

The extent of the potential hazard for consumers as a result of the TSE agent entering the meat following captive bolt stunning depends on the content of infectious prion proteins in the brain and hence on (a) the probability that an animal has been infected and (b) the incubation stage. Reliable estimates of infection probability are not possible given the current number of BSE cases in the country. However, since most cattle were obviously infected while still calves (Heynkes, 2001). Most BSE cattle were infected as calves, <http://www.heynkes.com/eng/eng.htm>. If the diffusion of the pathogens from the intestines to the brain along the digestive tract takes a certain time, the risk also depends on the age of the slaughtered animal. Therefore, it is taken into account by specifying the age limit for the removal of the CN (as SRM for cattle aged over 12 months).

According to the SSC (Effect of dissemination of brain particles into the blood and carcass when applying captive bolt stunning methods; SSC report of 10-11 January 2002, Part II, VII.4), several risk (GBR) levels can be distinguished as regards contamination of meat with the TSE agent following captive bolt stunning. For GBR level III countries (including Germany) the SSC recommends an age limit of 30 months for cattle. The SSC recognises that the risk is lower for cattle aged under 30 months, and higher in the case of cattle aged over 30 months. This estimate is based on the result of infectivity tests in which infection was not found in the brain of cattle infected as calves at the earliest 32 months subsequent to infection (C. S. GLAH, Hawkins, S.A.C., Green, R.B., Austin A.R., Dexter, I., Spencer, A.L., C. Glen, M.J., Stack, M.J. and Dawson, M., 1998: Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE) in cattle. *Vet Rec*, 142, S. 103-106) and the results of BSE tests of routinely slaughtered calves. However, the calves referred to by Wells et al. were not infected until they were 18 months old. Hence these tests suggest that the brain can be infected already after 18 months incubation period. However, under practical conditions

infection is possible already in first month of life (cf. Heynkes, 2001). Besides, BSE cases with clinical symptoms were also found in Germany even in animals aged under 30 months. The latest BSE case in Great Britain was even younger. In Great Britain during 1986-2002 there were at least 18 cases of BSE in animals aged under 30 months; the youngest animal was only 20 months old (<http://www.defra.gov.uk/animalhd/se/index.html>). Besides, the currently available BSE tests detect infection only at an advanced stage of incubation, and at the earliest six months before outbreak of the clinical disease. Currently one cannot reliably say how long after a BSE infection BSE pathogens will be detected in the brains of infected animals.

In a further opinion of 12 December 2001 (Report on TSE infectivity distribution in ruminant tissues - state of knowledge, 12 December 2001), the SSC indicates (Section III.3 - Factors associated with slaughter process) in particular the possibility of contamination during the transport of bovine head.

Besides the possibility of contamination with brain material released through the frontal hole in the skull caused by bolt stunning there is a very high likelihood of the meat being contaminated with a BSE agent containing material released through the foramen magnum. The current practice of suspending the severed head by the nasal septum in such a way that contamination of the meat with blood, fluid and brain material from the foramen magnum is prevented, in our opinion is not in our opinion satisfactory. This also applied to heads which are skinned while still attached to the carcass and which are suspended for the purpose of synthesis. Since the shot hole and the foramen magnum are not on the same level, rather at right angles to one another on the head, there is no way of suspending the head in such a way that the spillage or leakage of fluid or CNS tissue fragments is prevented. The situation is aggravated because the shot hole equalises the pressure in the skull for the fluid to escape, whereas if there is only one aperture a certain amount of fluid must be released but then further release is prevented as a result of the increase in internal pressure.

One way of preventing contamination with the BSE agent, which several parties have proposed, is to use a stopper (e.g. stopper, plastic foam, etc.) to close both the frontal hole (caused by penetration of the skull during bolt stunning) and the foramen magnum. This procedure might be possible since the contamination of cheek meat with brain material. However, the stopper used must have to be impermeable immediately after insertion into the hole of the skull and also survive the skinning process unscathed. While in extreme conditions the use of a stopper would be effective if bolt stunning is correctly performed, i.e. if the relatively small and circular aperture (the presence of hair on the neck and the or less ruling out other seals) - there is no guarantee that stunning will be performed properly.

Besides, because of the anatomical conditions of the foramen magnum it may be difficult to find an impermeable and durable stopper. Another problem will be the sealing of the frontal sinus, which can connect with the nasal cavity. As a result, the stopper must be able to withstand difficult transport conditions and must also be able to withstand so that BSE samples can be taken in cases when sampling is not possible after severing the head.

The attached table provides details on individual critical hygiene points in connection with slaughter, transport, BSE sampling and meat harvesting of bovine heads. The risks associated with current practices are set out and consequences and possible remedies proposed.

Summing up, the procedures presented for reducing contamination of cheek meat by the BSE agent are of a provisional nature and their effectiveness will have to put to the test. Besides, even with the aid of the proposed measures and the restriction of cheek meat extraction to cattle of less than 30 months of age, contamination of meat cannot be fully ruled out.

In our view, the following procedures for reducing contamination of cheek meat of cattle are appropriate as an interim proposal:

- harvest only the masseters;
- harvest only meat from animals less than 30 months of age;
- stunning should not be performed with a penetrating bolt (this is probably the most important measure, because opening the cranial cavity not only creates another aperture through which CNS material can escape but also equalises pressure in the cranial cavity, facilitating the release of CNS fragments and fluid);
- removal of head section and spine and spinal marrow using separate knives ("green", "brown", "saw" technique) – infect the utensils in separate sterilisation basins;
- remove horns without opening the cranial cavity;
- mechanically remove the brain from the head;
- directly immerse the head in a "closed" cleaning chamber, suspended (nose upwards) without contact with liquid;
- take BSE sample immediately after removal of head followed by sealing of the foramen magnum;
- transport and further processing in slaughterhouse with head suspended from the snout or corner of lower jaw;
- handle only in plastic protective clothing;
- clean only in a closed (under pressure) with underpressure;
- avoid all other treatment, including external washing;
- avoid use of water under pressure when cleaning;
- do not remove eyes;
- avoid all unnecessary contact with head;
- use separate knives ("green", "brown") when cutting the tongue and removing the tonsillar ring.

- always use fresh knife when inspecting the meat. First apply incisions to the external masseters, then remove tonsils,
- in the case of animals to be tested for BSE: do not use the so-called water or pressurised air method, take samples from head in suspended position; as regards sampling utensils (spoon, "cork borer") ensure that effective disinfection equipment is present (such as sterilisation basin with hypochlorite solution);
- when storing and transporting heads ensure that they are not suspended over one another and that they do not touch one another; ideally heads should not be transported at all,
- transport heads to cutting plant suspended side by side without touching one another.

In the interests of preventive consumer protection, if these measures should fail to prevent contamination of edible meat with CNS tissue the derogation should not be availed of and, in the case of a derogation over 12 months, the entire head without tongue, but with the brain, eyes, trigeminal ganglion and tonsils, should be classified as specified risk material.

Table: Critical stages in the harvesting and processing of bovine heads during slaughter, transport and harvesting of meat

| Critical hygiene point | Risks | Consequences/possible remedies |
|--|---|--|
| 1) Captive bolt stunning | Contamination of adjacent skin and slaughter area Contamination of the environs of the shot hole following removal of skin → Cross contamination hazard | - Avoid penetrative stunning |
| 2) Removal of horns | Opening of the cranial cavity → Cross contamination hazard | - Horn protect should not be applied lower than the base of the horn; avoid opening cranial cavity - If cranial cavity is opened : no further handling of the head |
| 3) Removal of head The use of a saw to cut the head off the carcass is also used on the carcass | Opening of the cranial cavity Contamination of the exposed areas of meat on the neck with the released fluid and the knives contaminated with spinal cord tissue → Cross contamination hazard | - Sever the vertebral canal as final stage in removal of head after severing the soft parts - Use fresh knife for every carcass and ensure BSE disinfection of the knives used before reuse (ensure that there is a suitable number of single-use knives for the batch) - Remove contaminated meat areas after sealing the foramen magnum (also with single-use knives) - Use separate knife to remove head and for further handling of head - Use separate sterilisation basin for this knife only - Seal perforated cranium (bolt shot hole and foramen magnum) to reduce contamination |

| Critical hygiene point | Risks | Consequences/possible remedies |
|---|---|---|
| 4) Suspension of head | Contamination of the exposed areas of meat on the neck as a result of released fluid and spinal cord particles | - Contamination-free transport and suspension of head |
| <u>5) Processing of head</u> | When working on tables contamination of surfaces with released CNS fluid is unavoidable → Cross contamination hazard | - Work only on suspended head - Avoid all handling which is not essential for harvesting the cheek meat (meat inspection) |
| After cleaning, the head is placed on the organ conveyor belt (transport to meat inspection point at end of slaughter line) | (with blood/brain/spinal cord particles) is not collected separately and removed, but flows over floor → spread of contamination by staff, when high-pressure cleaners are used walls and ceilings are contaminated; during transport on conveyor belt liquid drips off and spills onto the floor During work on tables, contamination of table surfaces with released CNS/fluid unavoidable → Cross contamination hazard | - Avoid external washing (this will require hygienic removal and skinning of head) - Ideally, soiled head-cleaning chamber (in EU-25, this has largely been realised), meat inspection of head (incisions to external masseters, etc.) at this place |

| Critical hygiene point | Risks | Consequences/possible remedies |
|--|--|--|
| 7) Cleaning of head (internal washing) | <p>When head is shifted, contamination hazard as a result of the release of fluid/CNS material from bolt shot hole and/or foramen magnum</p> <p>Brain particles may flow into the nasal cavity through bolt shot hole (the nasal cavity being connected to the brain via the cranial cavity)</p> <p>→ Cross contamination hazard</p> | <ul style="list-style-type: none"> - Avoid penetrative stunning - Avoid re-positioning of head |
| <p>Sampling is performed using sampling instruments (special spoons) or by applying pressure through the hole in the front of the cranium caused by bolt stunning, as a result of which the caudal brain regions are forced out through the foramen magnum</p> | <p>Contamination with BSE risk has been contaminated with spinal cord tissue and fluid following sampling</p> <p>Brain and fluid are deposited on the meat; this applies in particular in the cases of removal techniques in which the brain is forced out of the cranium</p> <p>→ Cross contamination hazard</p> | <p>For each animal first apply the BSE sampling instrument</p> <p>Take BSE sample immediately after severing the head and before sealing the foramen magnum</p> <ul style="list-style-type: none"> - BSE sampling and position should be so designed as to rule out contamination of the meat with brain material - Avoid sampling techniques in which the brain is forced through the cranium - Take BSE sample from suspended head, if possible before final severing of the head |

| Critical hygiene point | Risks | Consequences/possible remedies |
|--|---|---|
| 10) Transport of head from slaughterhouse to cutting plant Transport of bovine heads on so-called Christmas trees or in several layers suspended from a hook or in cages | Release of CNS and fluid with brain particles as a result of shaking during transport Contamination of lower heads by upper heads Spinal cord protein is released together with the fluid from the frontal bolt shot hole and from the foramen magnum Incomplete sealing of the frontal bore shot hole and the foramen magnum Transport of heads on so-called Christmas trees (heads stacked on top of each other) or in cages (heads stacked in several layers) leads to the release of brain material → Cross contamination hazard | <ul style="list-style-type: none"> - Check inter alia that the frontal bolt shot hole and the foramen magnum are thoroughly sealed - Remove visibly contaminated bovine heads from the batch - Avoid use of so-called Christmas trees and cages/do not transport heads in several layers suspended from hooks - Transport heads suspended side by side but not touching one another - Collect heads by age classes in order to avoid cross contamination |
| 11) Removal of eyes, removal of mouth etc.. | Exposure of optic nerves and hence creation of new apertures in cranium Contamination hazard when head is shifted Contamination hazard through contaminated hands → Cross contamination hazard | <ul style="list-style-type: none"> - Avoid removal of eyes and all further handling |
| 12) Cutting of head to harvest cheek meat Fixing of head and manual removal of meat from head | Spinal cord protein (possibly containing BSE prions) sticks to the knife; hot water in the sterilisation basin denaturises the protein but does not inactivate the BSE pathogen → Cross contamination hazard | <ul style="list-style-type: none"> - Thorough (mechanical) cleaning of knife before hot-water treatment - Treatment of heads in batches by age classes (<12 months; 12 to 24 months; > 24 months). |

